Intact Analysis of Biopharmaceuticals by Hydrophobic Interaction/Reversed Phase 2D-LC/MS System

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Introduction

Hydrophobic Interaction chromatography (HIC) is a popular LC technique widely used in downstream process purification but recently gained interest in analytical scale analysis of mAbs and ADCs. HIC separates and purifies protein molecules based on their hydrophobicity, but unlike traditional reversed phase chromatography, it uses non- denaturing mobile phase solutions to preserve the biological activity of the intact proteins HIC technique can be applied to separate mAb variants such as oxidation and isomerization species, which are often different to isolate from other orthogonal chromatography methods. It also a popular tool in determining drug-to-antibody ratio (DAR) value which is considered quality assessment of an ADC product. Such investigations often benefit from mass measurement using Mass Spectrum (MS) detection, but the high salt conditions used for HIC separations make it incompatible to couple online MS.

In this presentation, we present 2D-LC approach to overcome this obstacle, which affords multiple heart-cutting (MHC) and subsequent desalting/separation using reversed-phase chromatography on-line with TOF MS were explored.

Experimental

Sample Preparation

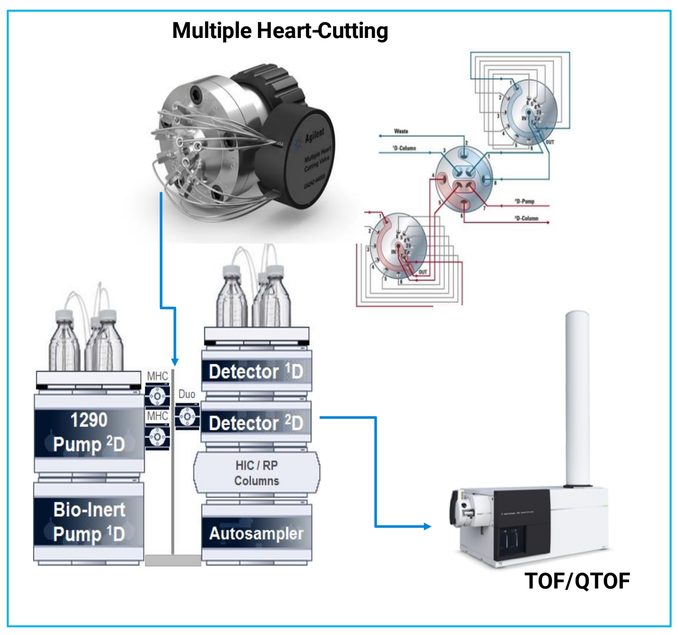
Mobile phases were prepared by diluting a stock salt solution (as indicated) with water (for Mobile Phase A and B). Both solutions were adjusted with 50% sodium hydroxide solution to pH 7. All samples were diluted with mobile phase B at a 1:1 volume ratio to avoid injection viscosity differences.

Oxidation Conditions

mAb sample used in 2D-LC/MS application was subject to forced oxidation using 1% tert-Butyl Hydro peroxide (*t*-BHP) for 72 hours at 37 °C.

Instrumentation

Samples were analyzed on the Agilent 1290 Infinity 2D-LC Solution using an Agilent 1260 Infinity II Bio inert LC system on [1]D and 1290 Infinity II LC system on [2]D coupled with an Agilent 6224 TOF LC/MS system with dual-nebulizer ESI source. Figure 1. below illustrates the instrument setup..



Data Analysis

Data was analyzed using Agilent Open LAB CDS ChemStation Edition Rev. C.01.07 and Agilent Mass Hunter workstation software, Bio confirm version B.07.01.

Experimental

LC Conditions – HIC [1]Dimension parameters	
Column	Agilent AdvanceBio HIC, 4.6 x 100 mm 3.5 µm, P/N 685975-908
Column Temp	25 °C
DAD	280 nm
Injection Vol.	1 -10 µL
Auto sampler Temp	4 °C
Mobile Phase	A = 50 mM Sodium Phosphate pH 7.0 B = 2 M Ammonium Tartrate in A pH 7.0 C = Isopropanol
[1]D Flow Rate	0.4 mL/min

	Project 1			Project 2			
	Time	**% A**	**%B**	**Time**	**%A**	**%B**	**%C**
Gradient Program	2	25	75	0	25	75	0
	17	100	0	15	75	0	25
	20	100	0	20	75	0	25
	22	25	75	21	25	75	0
	25	25	75	25	25	75	0

LC Conditions – RP [2]Dimension Parameters	
Column	Agilent AdvanceBio RP-mAb C4, 2.1 x 50 mm, P/N 799775-904
Column Temp	80 °C
[2]D Flow Rate	0.2 mL/min
2D-LC mode	Multiple heart-cutting
[2]D Gradient stop	5 minutes
[2]D cycle time	7 minutes
Mobile Phase	A = 0.5% Acetic acid, 0.05% TFA in water B = 0.5% Acetic acid, 0.05% TFA in 80:10:10 acetonitrile/1-propanol/water
Valve and loop configuration	2- position / 4-port-duo 2x6 loops (concurrent); Loop size: 40 µL
Gradient Program	**Time** **% B** 2 28 4 42 4.6 50

Results and Discussion

Project 1: HIC/RP-2D-LC/MS Analysis of Oxidized mAb

In HIC separations, proteins interact with a weakly hydrophobic stationary phase in the presence of a high initial concentration of lyotropic or cosmotropic salts. The concentration of this salt is lowered as the gradient proceeds, resulting in elution of the protein from the stationary phase.

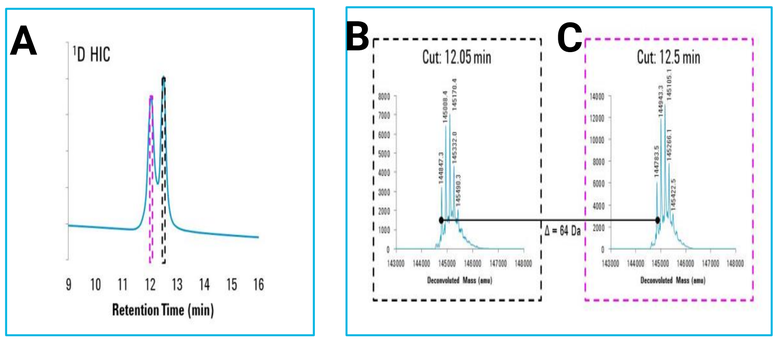
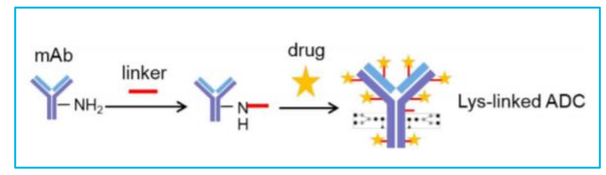


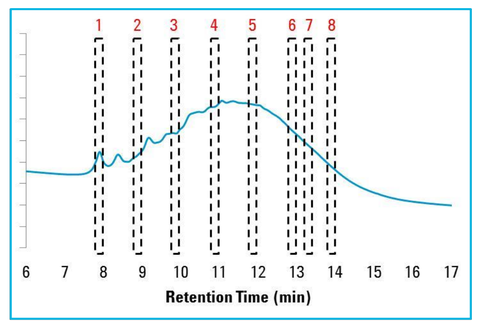
Figure 3. Analysis of Forced Oxidized mAb. (A) HIC chromatogram of mAb sample treated with 1 % *t*-BHP. *t*-BHP treatment resulted in a new peak at RT 12.05 minutes. (B) Mass measurement of the peak at 12.05 minutes. (C) Mass measurement of the peak at 12.5 minutes. The mass of the oxidized mAb was 64 Da higher than the untreated mAb, indicating four potential sites of oxidation.

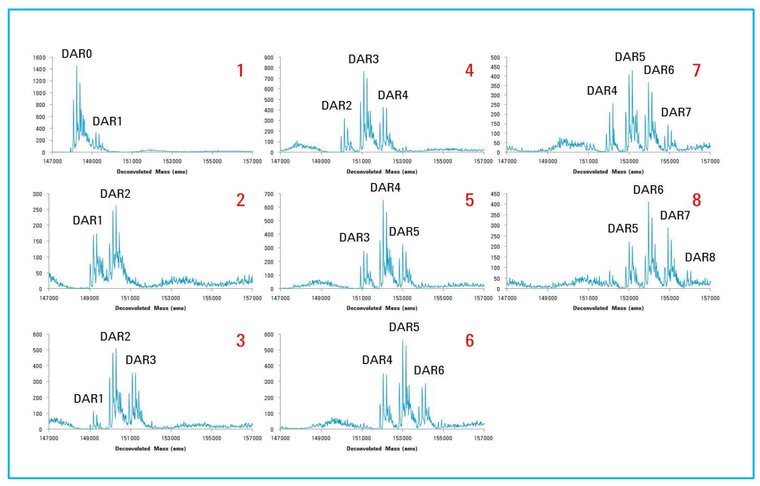
Results and Discussion

Project 2: HIC/RP-2D-LC/MS Analysis of Lys-linked ADC

Figure 3. A Lysine-linked ADC was analyzed using the HIC/RP 2D-LC/MS system. Lys-linked ADC sample is illustrated as cartoon below. No sample deglycosylation was performed prior to analysis. The resulting HIC chromatogram consisted of a very poorly resolved group of peaks eluting over the course of ~7 minutes, reflective of the multiple positional isomers present in the preparation. Eight heart-cuts at RTs 7.9, 8.9, 9.9, 10.9, 11.9, 12.9, 13.4, and 13.9 min were selected and sent to the [2]Dimension Reverse phase separation to TOF MS.







The MS spectra corresponding to the heart-cuts are shown above. mAbs with DAR ranging from 0-8 were observed. The deconvoluted mass spectra revealed that RT increases as a function of DAR, as expected. It is also evident, based on the fact that certain DAR species are present in multiple heart-cuts, that there is some selectivity based on the positional isomers in the mixture. Species containing between 0 (unconjugated mAb) and a maximum of 8 small molecule drugs were detected. The spectra also reflect the glycoform distribution of the mAb in addition to a subpopulation of molecules modified by linker without payload.

Conclusions

- AdvanceBio HIC column proves to be an incredibly useful tool for characterizing mAbs, even when modified components are present as minor species.
- Use of high salt concentration mobile phases are required for HIC separations, this limits its use in characterization with Mass spec. AdvanceBio HIC column coupled with Agilent Infinity II Bio-Inert LC and 2D-LC workflows presented here help resolve these issues and provide an easy solution for routine characterization of large bio-molecules.

References

Staples, G. Analysis of monoclonal antibodies using MHC 2D-LC/MS.. Agilent Technologies, Application Note. 2014. (5991-6376EN)

Learn more about Agilent AdvanceBio columns
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